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TECHNICAL MANUSCRIPT 210

ATTENUATED TOTAL REFLECTANCE IN INFRARED SPECTROPHOTOMETRY OF BIOLOGICAL SYSTEMS

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UNITED STATES ARMY
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TECHNICAL MANUSCRIPT 210

ATTENUATED TOTAL REFLECTANCE IN INFRARED SPECTROPHOTOMETRY OF BIOLOGICAL SYSTEMS

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ABSTRACT

The attenuated total reflectance (ATR) method for obtaining infrared absorption spectra has been shown to be a feasible technique for the study of biological and other materials in their normal aqueous environment. Water-compensated ATR spectra for a number of simple compounds, a protein (bovine serum albumin), and a bacterium (*Serratia marcescens*) in water solution or suspension, were generally similar to normal transmission spectra for dried samples; but showed some distinctive features not present in the latter. Some limitations of the method and steps taken to overcome them are discussed.

I. INTRODUCTION

Infrared spectral measurements by conventional transmittance methods have been of very limited usefulness in the study of biological and other materials that normally exist in an aqueous milieu. The intense water absorption bands severely limit the spectral range in which useful infrared data can be obtained. The usual procedure with these materials has consisted of either preparing dried films on silver chloride plates, or compressing a mixture of the dried sample with potassium bromide powder into disk form. Although this permits infrared data by transmittance measurements to be obtained without interference by water absorption, the results may be unsatisfactory for one or more of the following reasons: (i) additional absorption bands due to the crystalline state can be confused with fundamental vibrations, (ii) quantitative measurements are frustrated by the difficulty in controlling the thickness of transmission layers, and (iii) the removal of water precludes the study of structural properties and kinetic phenomena that are characteristic of aqueous systems.

As one solution to this problem, Gore et al.* suggested using deuterium oxide as a solvent. The latter is more transparent than water particularly at wavelengths of greatest interest because the O-D stretching and bending frequencies are considerably lower than the corresponding O-H frequencies. However there is much evidence that the hydrogen-deuterium exchange produces structural and functional abnormalities in biological systems.

A very promising alternative to conventional transmittance methods was offered when Fahrenfort**developed the attenuated total reflectance (ATR) technique. In this method reflection spectra are obtained by employing the interface between a dielectric of high refractive index and the sample as the reflecting surface. If the incident beam at this interface is at an angle larger than the critical angle it will be totally reflected, but only in those frequency regions where the sample is nonabsorbing. At absorbing frequencies reflection will not be total, and in this way, a reflection spectrum is obtained that strongly resembles a transmission spectrum. The ATR spectra involve an extremely shallow penetration (less than 5 microns) into the sample and are independent of sample thickness.

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* Gore, R.C., R.B. Barnes, and E. Peterson. 1949. Infrared absorption of aqueous solutions of organic acids and their salts. Anal. Chem. 21:382.

** Fahrenfort, J. 1961. Attenuated total reflection. A new principle for the production of useful infrared reflection spectra of organic compounds. Spectrochim. Acta 17:698-709.

The ATR technique has already affected the entire field of infrared spectrophotometry, for it provides a simple procedure for obtaining infrared spectra with almost any material and with virtually no sample preparation. However it has been applied mostly to solid samples. Our purpose has been to investigate the usefulness of the ATR technique for aqueous biological systems such as bacterial and viral suspensions and protein solutions. Because the effective penetration of the beam into the sample is only a few microns, one should be able to compensate for water absorption in a double beam instrument.

II. EXPERIMENTAL

A double beam recording infrared spectrophotometer (Perkin-Elmer Model 21) has been used in this study. The required changes in optical arrangement included (i) a means for providing an adjustable angle of incidence of the infrared beam on the internally reflecting face of a prism; (ii) an infrared transmitting prism of higher refractive index than the sample to be examined; and (iii) a means of holding the sample in close contact with the prism. These changes were achieved by use of a pair of Connecticut Instrument Co. Model ATR-1 attachments, one for the sample beam and one for solvent compensation in the reference beam. Figure 1 shows a schematic diagram of the arrangement of the mirrors, prism, and sample holder which are contained in the attachment. At first KRS-5 prisms were used because their wide transmission range and refractive index (2.38) make them applicable to a broad variety of samples. However, it was later found that an apparent drift in absorption was caused by a gradual etching at the surface of the KRS-5 prism. Consequently, a pair of prisms made of Irtran -2 (Eastman Kodak Corp.) was acquired and is being used currently. Irtran -2 has a refractive index of 2.2 and is chemically inert to nearly all solvents. Its limitation is its inability to transmit beyond 11 microns.

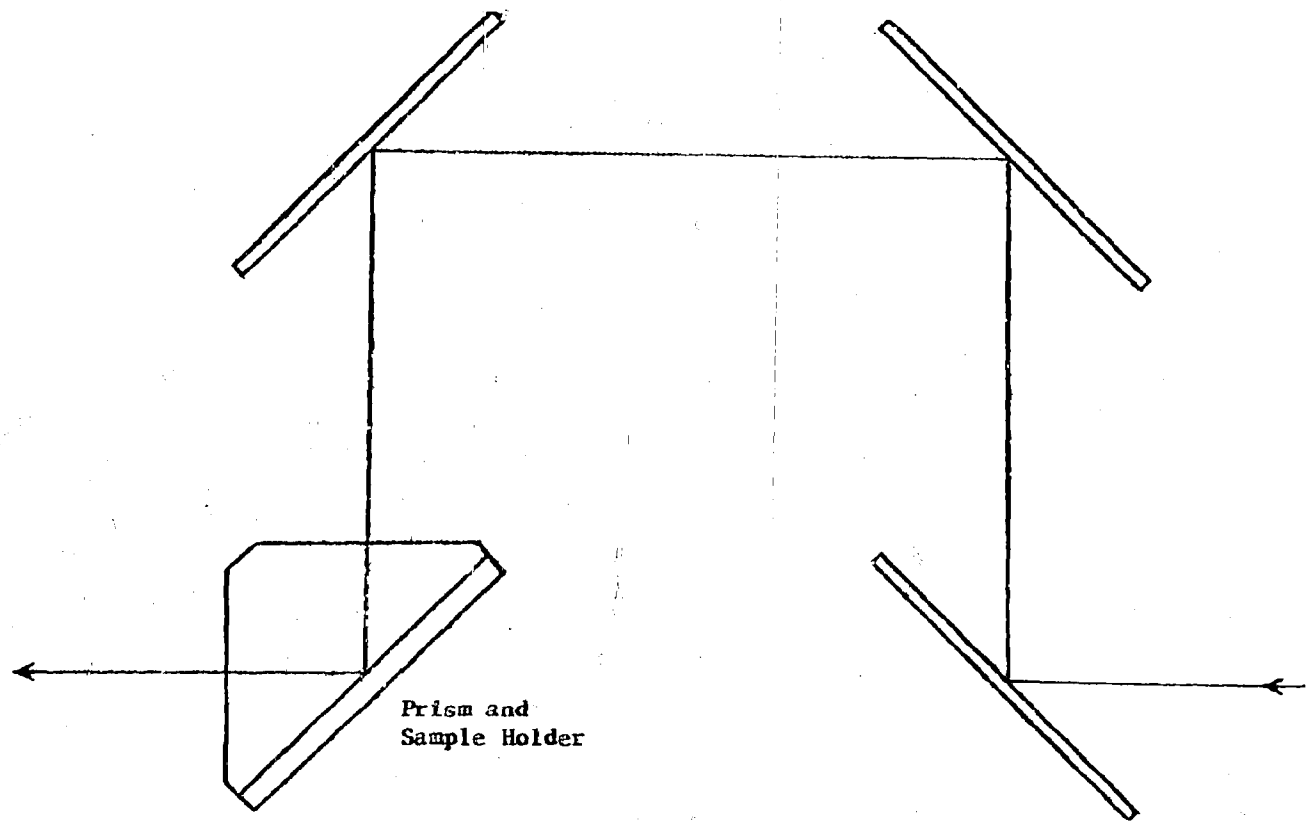

Figure 1. Arrangement of Mirrors, Prism and Sample Holder in Model ATR-1 Attachment. Angle of incidence can be varied from 30° to 75°.

III. RESULTS AND DISCUSSION

The practicability and validity of the ATR infrared method were first observed in measurements with several pure organic liquids including acetone, carbon tetrachloride, ethanol, dioxane, and trimethylpentane. As illustrated by the data for acetone in Figure 2, the ATR spectra with these liquids were nearly identical to the corresponding curves obtained by the transmission method using capillary cells. Subsequently, ATR spectra with various aqueous solutions were obtained; these involved compensation for water absorption by use of a reference ATR-1 unit. Figure 3 shows a comparison of the ATR spectra for pure acetone and 40% (v/v) acetone in water, respectively, in the structurally significant wavelength range of 5 to 9 microns. The interesting feature here is the shift of the carbonyl absorption peak at 5.9 microns to a longer wavelength accompanied by broadening of the band, apparently the result of intermolecular hydrogen bonding to water. Similarly, ATR spectra were obtained for a number of aqueous salt solutions, including dibasic potassium phosphate, sodium chloride, citrate buffers, and phosphate buffers. These were more definitive than the corresponding transmission spectra derived by either the film or KBr pressed-disk techniques, due to the elimination of solid state scattering.

The ATR spectra of bovine serum albumin (BSA, Fraction V, Nutritional Biochemicals) in the form of a dried film and in a 10% (w/w) aqueous solution are shown in Figure 4 along with a transmission spectrum of the dried BSA film. Only minor differences are seen in the spectra for the solid protein by the two techniques. However, important differences are noted in comparing the spectra for dissolved and undissolved BSA. For example, the C-H absorption bands at 3.5 and 7.0 microns are suppressed in aqueous solution and there is also a decreased intensity of the band at 6.2 microns relative to that at 6.5 microns, and of both of these relative to that at 3.2 microns. No explanations for these differences are offered at this time.

Figure 5 shows the ATR spectra, in the 6- to 9-micron interval, of washed S. marcescens bacteria in a 15% (w/w) aqueous suspension and in the form of a dried film. Similar absorption bands are observed in the two cases; however, in aqueous suspension, the bacteria show a pronounced decrease in the relative absorption of the band at 6.2 microns, a behavior observed earlier with dissolved BSA.

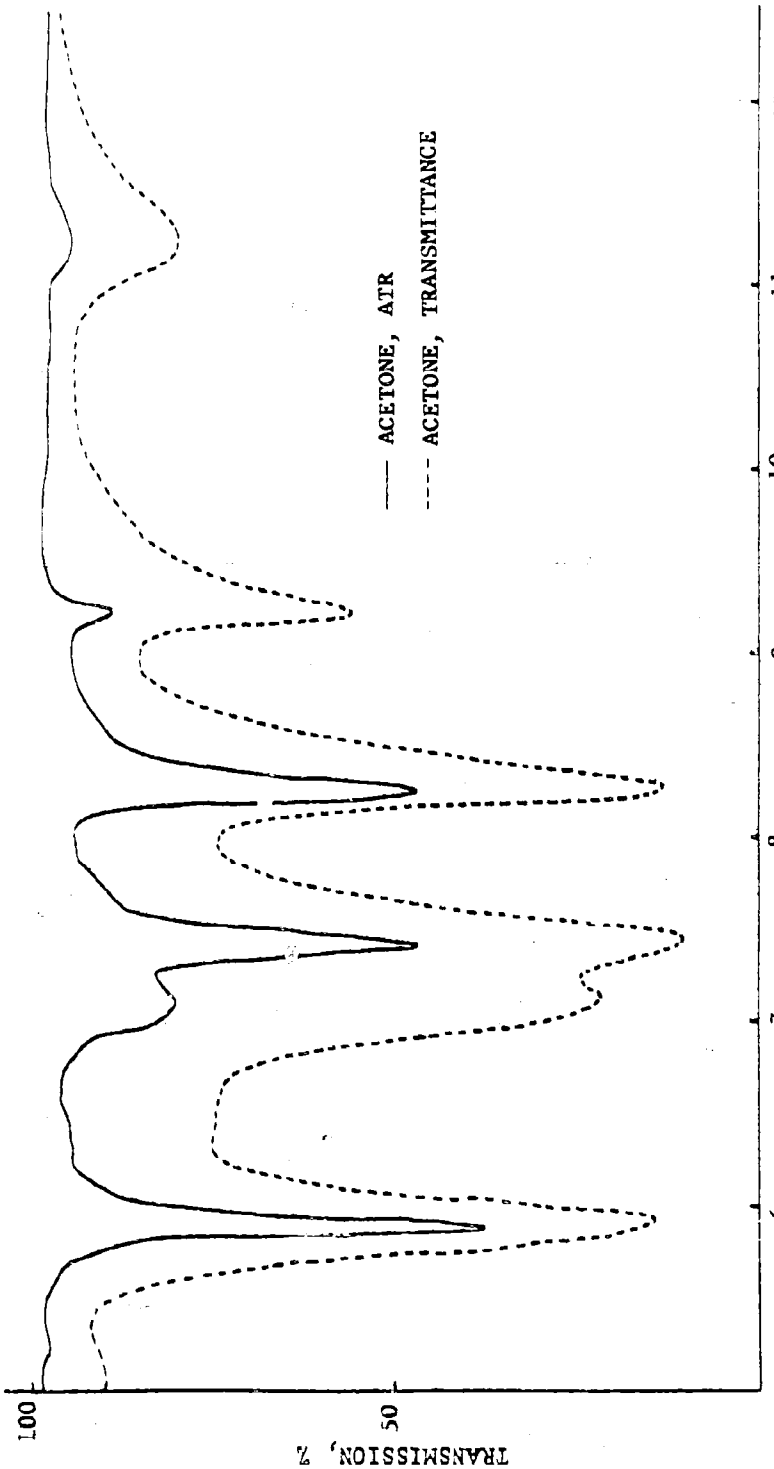

Figure 2. Comparison of Infrared Spectra of Acetone by ATR and Transmittance Methods. Prism, KRS-5; angle of incidence, 45°; transmittance in KRS-5 capillary cell.

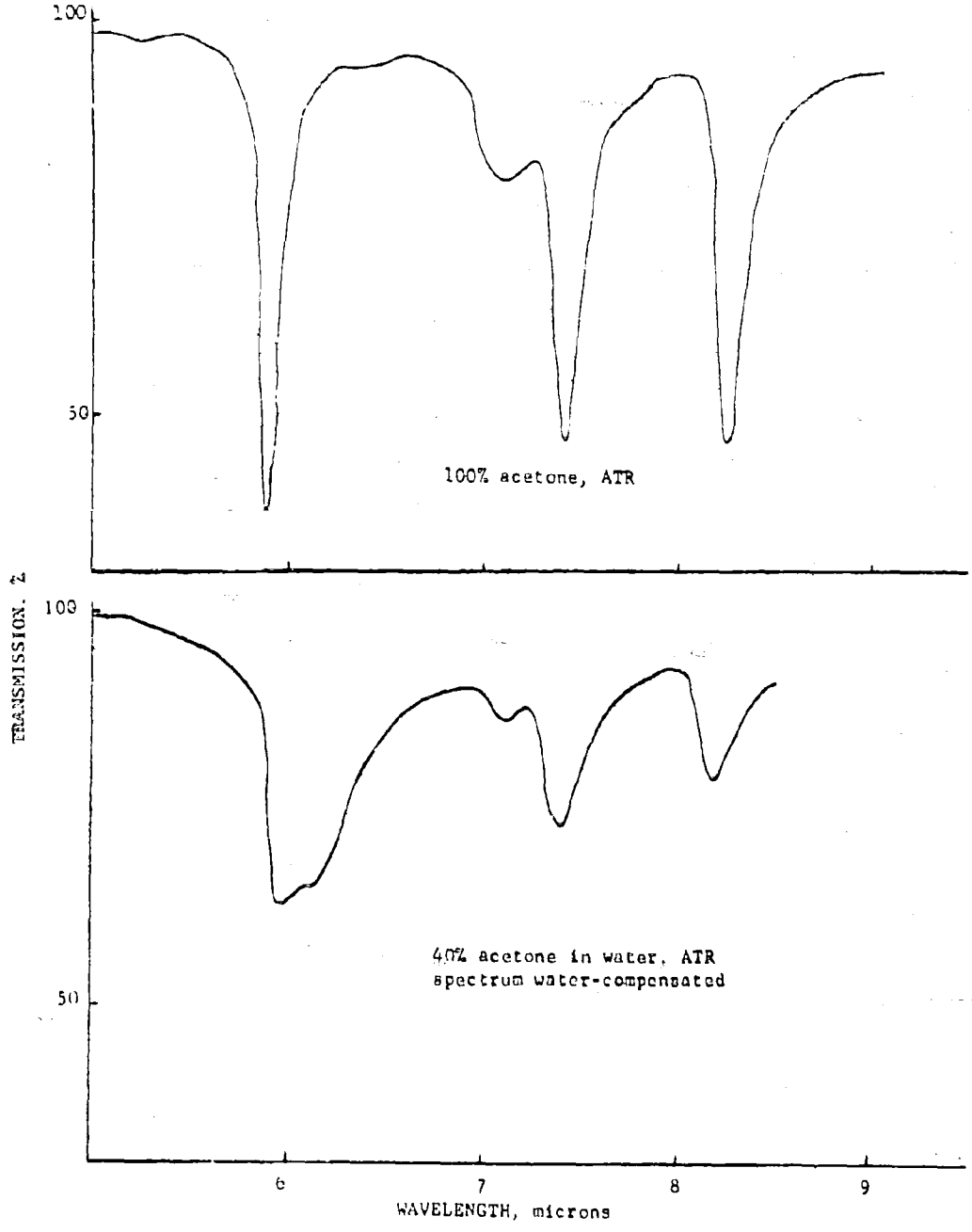

Figure 3. ATR Spectra of Pure Acetone and of 40% (v/v) Acetone in Water. Prism, KRS-5; angle of incidence, 45°.

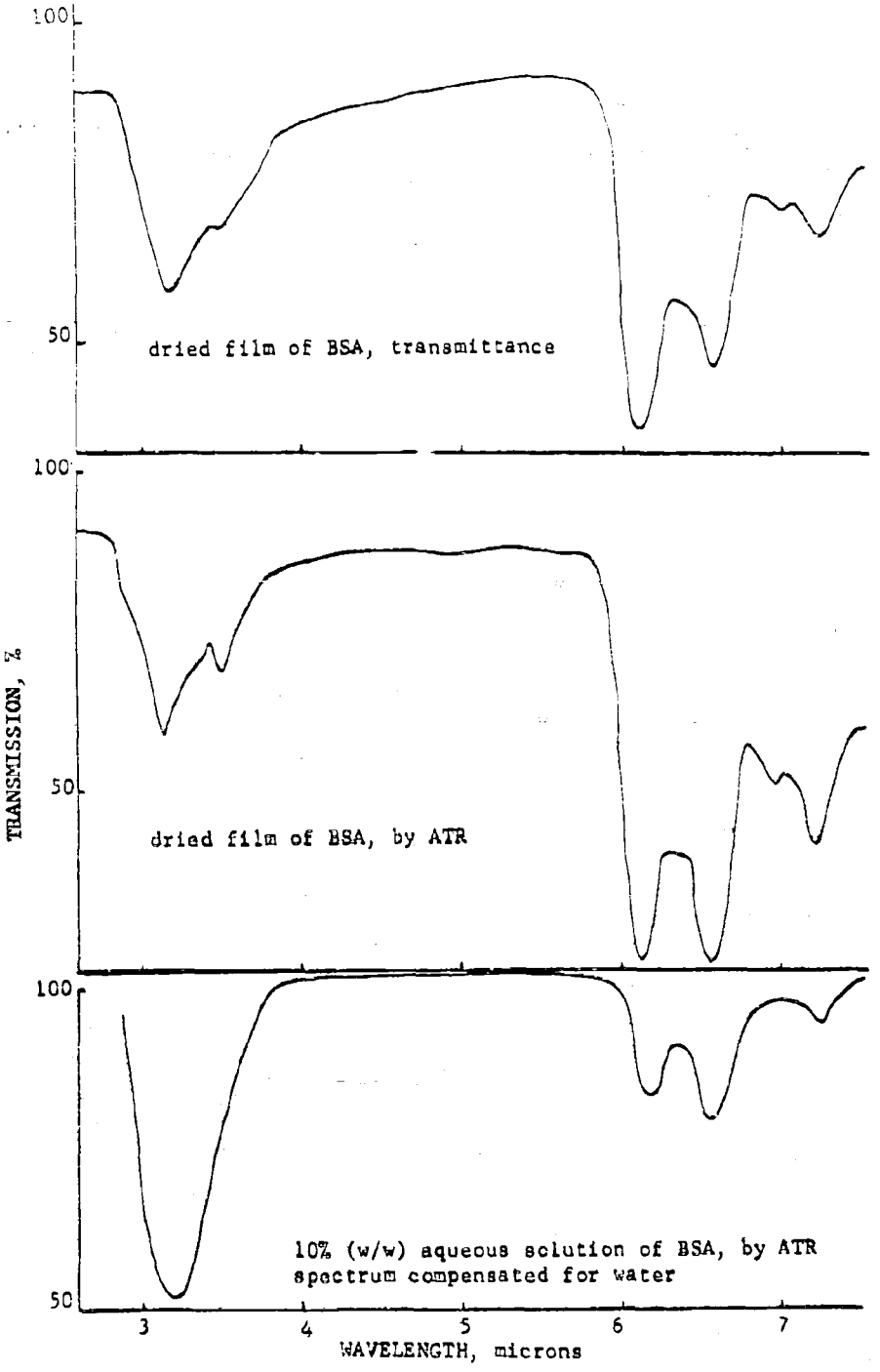

Figure 4. ATR Spectra of Dried and Dissolved Bovine Serum Albumin Compared with Transmittance Spectrum of Dried Sample.

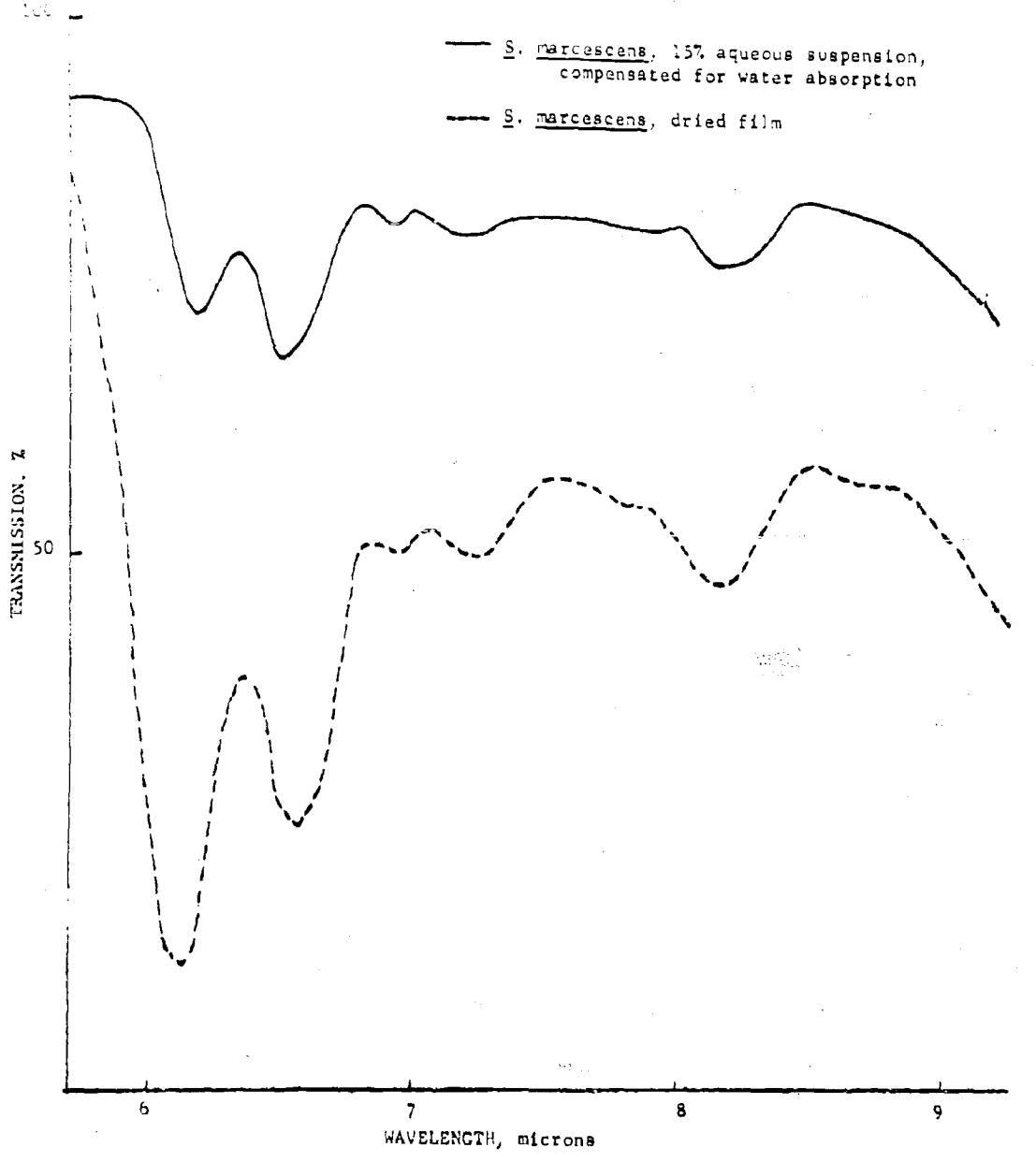

Figure 5. Comparison of ATR Spectra of S. marcescens in dried and Water-Suspended States. Prism, KRS-5; angle of incidence, 45°.

Since the ATR method involves only a very short effective path length, high concentrations of material (10% or higher) in aqueous solutions were required to obtain reasonably intense spectra. This is a serious limitation, and an attempt to overcome it was made by equipping the spectrophotometer with an ordinate scale expander (up to 20X). This device permits increased sensitivity without an appreciable increase in the noise-to-signal ratio. An alternative to this, one which we have not used, is an arrangement employing multiple reflections.

In using scale expansion to obtain ATR spectra with water or with aqueous solutions, an effect was observed which had not been noted previously. At scale expansions of 5X or higher, there was constant and quite rapid drifting toward increased absorption over a broad range of wavelengths, an effect which prevented the attainment of reproducible spectra. The difficulty was traced and found to be caused by etching of the reflecting surface of the KRS-5 prism by water, for the effect could be reversed by repolishing this surface. As shown in Figure 6, the drift is also detectable without scale expansion, but only over a much longer time interval. Thus in order to permit use of scale expansion with aqueous solutions, it became necessary to replace the KRS-5 prisms with a pair of the more chemically inert Irtran -2 prisms, as mentioned earlier. However, the KRS-5 prisms will continue to be more useful with solid or nonaqueous liquid samples because of their broader transmission range.

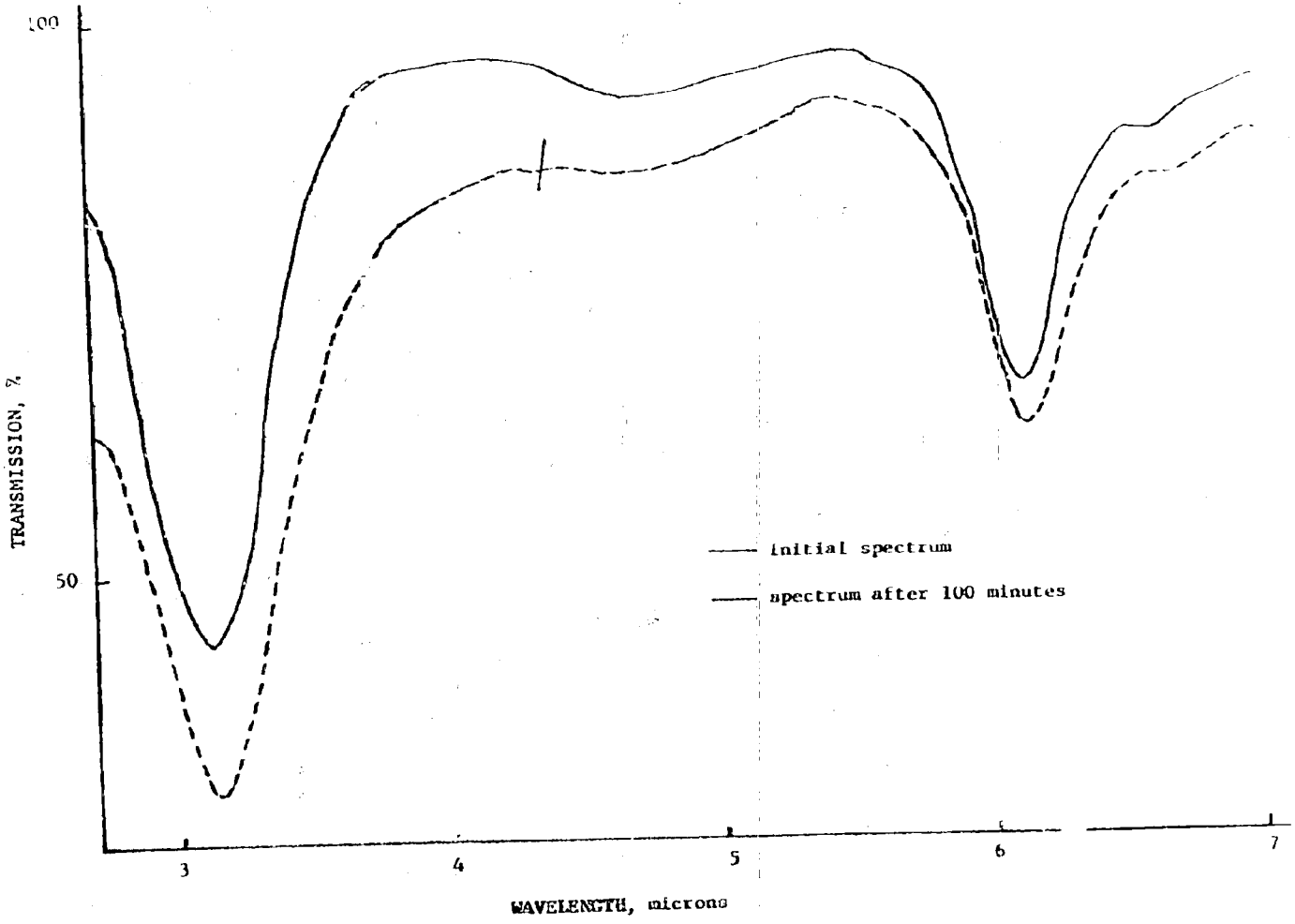

Figure 6. Drift of ATR Spectrum of Water Using KRS-5 Prism.

IV. CONCLUSIONS

Experiments with the ATR method show it to be a practicable technique for obtaining useful infrared spectra with materials in aqueous solution or suspension. This is of major importance for the study of biological systems, for it provides a simple and attractive way of avoiding the necessity for drying samples, thereby making structural and metabolic phenomena in such systems more amenable to analysis by infrared spectrophotometry. ATR spectra of a variety of substances, including simple compounds, a protein (BSA), and a bacterium (S. marcescens) dissolved or suspended in water, demonstrate some distinctive features not present in spectra with dried samples.

A limitation in using the ATR technique with standard instruments is the need for high concentrations of materials to obtain absorption bands of sufficient intensity. To overcome this, the Perkin-Elmer Model 21 in this laboratory has been equipped with an ordinate scale expander. Irtran -2 prisms are now being used with water solutions because of their chemical inertness, but for samples that do not attack KRS-5 prisms, the latter are more useful because of their broader transmission range.

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